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Enzymatic profiles of selected therm philic actinomycetes

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Abstract

Few data are presently available regarding the enzymatic profiles of the thermophilic actinomycetes. In an attempt to examine more fully the enzymatic capabilities of these organisms API ZYM strips were used to assay for the presence or absence of nineteen different enzymes. Culture supernatants (DDA) obtained from thirteen isolates of *Thermoactinomyces candidus,* ten isolates of *T. vulgaris,* two isolates of *T. sacchari,* seven isolates of *Micropolyspora faeni,* four isolates of *Saccharomonospora viridis,* and four isolates of *Thermomonospora fusca,* were assayed. Whole cells from selected isolates were also assayed. Alkaline and acid phosphatase, C4 esterase, and C8 esterase-lipase activities were demonstrated for both whole cells and DDA of *T. candidus. M. faeni* whole cells and DDA contained alkaline and acid phosphatase, phosphoamidase, C4 and C8 esterase-lipase, leucine and cystine aminopeptidase activities. Whole cells of *T. vulgaris* displayed C4 esterase, leucine aminopeptidase, chymotrypsin, and α-glucosidase activities whereas DDA contained only phosphoamidase and α-glucosidase activity. Culture supernatants of *T. sacchari* showed alkaline phosphatase, C4 and C8 esterase-lipase activities. *T. fusca* supernatants contained C4 and C8 esterase-lipase activities, leucine aminopeptidase, β-galactosidase, and α-glucosidase activities. *S. viridis* had alkaline phosphatase, C4 and C8 esterase-lipase, C14 lipase, leucine aminopeptidase, α-glucosidase and N-acetyl-β-glucosaminidase activities. The differences in enzymatic profiles for these actinomycetes allowed clear differentiation between genera and species. The API ZYM system offers an easy and rapid assay system for nineteen different enzymes simultaneously.

Introduction

The thermophilic actinomycetes have been implicated as sensitizing agents in the development of hypersensitivity pneumonitis (Burke *et al.,* 1977; Fink *et al.,* 1976; Pepys, 1969). Several studies have suggested that sensitization and disease production may occur after inhalation of a combination of enzymes, spores, and mycelial fragments from species of *Thermoactinomyces, Saccharomonospora,* and most notably *Micropolyspora faeni* (Nicolet and Bannerman, 1975; Nicolet, Bannerman and Krawinkler, 1974; Schallibaum *et al.,* 1977). In the course of investigating cases of hypersensitivity pneumonitis we have been isolating and identifying thermophilic actinomycetes. Identification is facilitated by use of the morphological and biochemical criteria as outlined by Kurup and Fink (1975). However, differentiation between *Thermoactinomyces candidus, T. vulgaris,* and *Thermomonospora fusca* can be difficult and may require 1–2 weeks before an identification can be made (Kurup, 1979; Kurup and Fink, 1975). In addition, many of the biochemical tests needed are not available in clinical laboratories. Since *T. fusca* has not been associated with hypersensitivity pneumonitis (Kurup, 1979) as has *T. candidus* and *T. vulgaris* a rapid and simple method for preliminary differentiation would be of value.

Several reports have recently appeared which indicate that determination of enzymatic profiles can be useful for identification purposes (Hofstad, 1980; Humber, King and Phillips, 1977; Kilian, 1978; Waitkins, Ball and Fraser, 1980). The determination of the enzymatic profiles in these reports was facilitated by use of the commercially available API-ZYM strips. Studies have appeared which deal with the enzymes produced by selected thermophilic actinomycetes (Bannerman and Nicolet, 1976; Desai and Dhala, 1969; Elwan *et al.,* 1978 (a,b); Roberts *et al.,* 1977; Sinha

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and Singh, 1980). These reports, however, usually describe the isolation and physiochemical properties of one enzyme. Few papers have described attempts to assay for more than one enzyme (Kimura, Lopez and Salvaggio, 1975; Walbaum, Biguet and Tran van Ky, 1969). Therefore, the utility of enzyme profiles for classification of selected thermophilic actinomycetes using the API-ZYM system was assessed.

Materials and methods

Organisms

The source and identification of the thermophilic actinomycetes used in this study are listed in Table 1. Identification was based upon the biochemical reactions published by Kurup and Fink (1975). Organisms assayed for enzymatic activity (*T. candidus,* seven isolates; *T. vulgaris,* four isolates; and *M. faeni,* two isolates) were harvested from the outer phase of the double dialysis antigen (DDA) preparation (described below). The cells were washed three times in saline before resuspending to match a McFarland 5 standard.

Double dialysis antigen

DDA was prepared from all of the isolates according to previously described methods (Edwards, 1972), except that the second dialysis step was omitted. Briefly, 50 ml of tryptic soy broth or tryptic soy broth with 2% yeast extract for *T. sacchari* was placed in dialysis tubing (designated inner phase). The tubing was then placed into a flask containing 200 ml of 0.1 M glycine and 0.075 M NaCl (designated outer phase). The flasks were incubated at 50°C for 72 h before removing the dialysis tubing. Flasks were inoculated and incubated for 2 weeks at 50°C. Organisms were recovered by centrifugation and treated as described above. The supernatant fluid remaining after centrifugation constituted the DDA. Either neat or 5 x concentrated DDA was then assayed for enzymatic activity. Concentration was achieved with a Minicon B 15 concentrator (Amicon Corp., Lexington, Massachusetts, U.S.A.).

Assay of enzymatic activity

Enzymatic activity of whole organisms, DDA, and 5 x DDA was assayed using API ZYM strips (Analytab Products, Plainview, New York, U.S.A.). The nineteen enzymes detectable by this system are listed in Table 2. Strips were inoculated in duplicate according to the manufacturer's instructions and incubated for 4 h at 50°C. Selected isolates were also tested at 35°C. Enzymatic activity was visualized by the addition of reagents A [250 g Tris (hydroxymethyl) aminomethane, 110 ml hydrochloric acid (37%), 100 g laurylsulphate (sodium dodecyl sulphate), q.s. to 1,000 ml with distilled water] and B (3.5 g Fast Blue BB, q.s. 1,000 ml with 2-methoxyethanol) supplied with the strips. The reactions were quantitated as 0 to 5+ by comparison to a colour chart provided by the manufacturer. A 1+ reaction was considered positive. Uninoculated broth (prepared the same as DDA) and saline inoculated strips served as negative controls.

Table 1 S urce and identification of isolates

Cultures and designation when received*		Identification
VPK	T150, *Micropolyspora faeni* (ATCC-15347)	*M. faeni*
	T152, *M. faeni*	*M. faeni*
	T154, *M. faeni*	*M. faeni*
	T210, *M. faeni*	*M. faeni*
JHE	*M. faeni*	*M. faeni*
MJ	M18, *M. faeni*	*M. faeni*
JL	A94, *M. faeni*	*M. faeni*
MJ	*Saccharomonospora viridis*	*S. viridis*
JL	A66, *S. viridis*	*S. viridis*
UR	23, *S. viridis*	*S. viridis*
	80, *S. viridis*	*S. viridis*
UR	3647, *Thermonomospora fusca*	*T. fusca*
	3750, *T. fusca*	*T. fusca*
	3805, *T. fusca*	*T. fusca*
	3846, *T. fusca*	*T. fusca*
VPK	T106, *Thermoactinomyces candidus* (ATCC-7868)	*T. candidus*
	T115, *T. candidus*	*T. candidus*
JHE	*T. vulgaris*	*T. candidus*
	T. vulgaris,melanin negative	*T. candidus*
MJ	CUB250, *T. vulgaris*	*T. candidus*
UR	110, *T. vulgaris*	*T. candidus*
	316, *T. candidus*	*T. candidus*
	771, *T. candidus*	*T. candidus*
	972, *T. candidus*	*T. candidus*
	1222, *T. candidus*	*T. candidus*
	1259, *T. candidus*	*T. candidus*
	20, 138, *T. candidus*	*T. candidus*
	20, 139, *T. candidus*	*T. candidus*
VPK	T145, *Thermoactinomyces sacchari*	*T. sacchari*
JL	A978, *T. sacchari*	*T. sacchari*
VPK	T101, *Thermoactinomyces vulgaris*	*T. vulgaris*
	T126, *T. vulgaris*	*T. vulgaris*
	T156, *T. vulgaris*	*T. vulgaris*
	T167, *T. vulgaris*	*T. vulgaris*
JHE	*T. vulgaris*, melanin positive	*T. vulgaris*
MJ	CUB804, *T. thalpophilus*	*T. vulgaris*
	CUB808, *T. thalpophilus*	*T. vulgaris*
JL	A64, *T. thalpophilus*	*T. vulgaris*
UR	399-1, *T. vulgaris*	*T. vulgaris*
	399-2, *T. vulgaris*	*T. vulgaris*

* VPK — V. P. Kurup, Wood Veterans Administration Center, Wood, Wisconsin, U.S.A.; JHE — J. H. Edwards, Medical Research Council, Llandough Hospital, Penarth, England; MJ — M. J. Jones, Marshfield Medical Foundation, Marshfield, Wisconsin, U.S.A.; JL — J. Lacey, Rathamsted Experimental Station, Harpenden, Hertfordshire, England; UR — University of Rochester stock culture collection.

Table 2 Enzymes detected by the API-ZYM system

Test	Enzyme assayed	Test	Enzyme assayed
0	control	10	acid phosphatase
1	alkaline phosphatase	11	phosphoamidase
2	esterase (C4)	12	α-galactosidase
3	esterase-lipase (C8)	13	β-galactosidase
4	lipase (C14)	14	β-glucuronidase
5	leucine aminopeptidase	15	α-glucosidase
6	valine aminopeptidase	16	β-glucosidase
7	cystine aminopeptidase	17	N-acetyl-β-glucosaminidase
8	trypsin	18	α-mannosidase
9	chymotrypsin	19	α-fucosidase

Results

The results of the enzyme profiles are summarized in Table 3. None of the organisms which were tested yielded positive reactions in tests for valine aminopeptidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, or α-fucosidase. Saline and uninoculated broth controls consistently gave negative results in all tests.

Data presented in Table 3 are from unconcentrated double dialysis antigen with the strips incubated at 50°C. Concentration of the double dialysis antigen did not result in detection of additional enzymes (data not shown); however, it did serve to intensify the reactions obtained. Incubation of the strips at 35°C (Table 4) resulted in the loss of activity of several enzymes. For example acid phosphatase for *M. faeni* gave negative reactions at 35°C. Loss of activity for acid phosphatase was noted for some isolates of *T. candidus;* α-glucosidase activity for *T. vulgaris* was also lost at this temperature.

The enzymes detected in the double dialysis antigen were, with the exception of *T. vulgaris*, identical to those seen when using whole organisms. It was found that several additional enzymes were detected using whole cells of *T. vulgaris.* While only α-glucosidase and phosphoamidase activity was found in the double dialysis antigen, whole cells also contained C4 esterase, leucine aminopeptidase, and chymotrypsin activities.

T. sacchari showed alkaline phosphatase, C4 and C8 esterase-lipase activity. In this regard, the enzyme profile obtained was similar to that of *T. candidus* except for the negative acid phosphatase reaction. *S. viridis* yielded positive reactions for acid phosphatase, C4 and C8 esterase-lipase, C 14 lipase, leucine aminopeptidase, α-glucosidase, and N-acetyl-β-glucosaminidase. *T. fusca* yielded C4 and C8 esterase-lipase, leucine aminopeptidase, β-galactosidase, and α-glucosidase activities.

Figure 1 shows that, with a minimum of four enzymatic reactions, differentiation to the generic level is possible. *T. candidus* and *T. sacchari* cannot be separated by their enzymatic profiles so that other biochemical tests must be performed for identification.

Table 3 Enzyme profiles of thermophilic actinomycetes as detected by the API-ZYM system incubated at 50°C using DDA*

Enzyme detected	*T. candidus* (13)**	*T. sacchari* (2)	*T. vulgaris* (10)	*T. fusca* (4)	*S. viridis* (4)	*M. faeni* (7)
Alkaline phosphatase	+	+	–	–	+	+
C4 esterase	+	+	–	+	+	+
C8 esterase-lipase	+	+	–	+	+	+
C14 lipase	–	–	–	–	+	–
Leucine aminopeptidase	–	–	–	+	+	+
Cystine aminopeptidase	–	–	–	–	–	±
Trypsin	–	–	–	–	–	+
Acid phosphatase	±	–	–	–	–	+
Phosphoamidase	–	–	±	–	–	+
β-galactosidase	–	–	–	+	–	±
α-glucosidase	–	–	+	+	±	–
N-acetyl-β-glucosaminidase	–	–	–	–	+	–

* Double dialysis antigen.
** Number of isolates tested.

Discussion

Using the API ZYM strip the presence or absence of nineteen different enzymes in selected thermophilic actinomycetes was assessed. The double dialysis antigen of *T. vulgaris* was found to contain phosphoamidase and α-glucosidase activities while assay of the whole organism showed C4 esterase, leucine aminopeptidase, and chymotrypsin activity. Other investigators have previously reported the presence of chymotrypsin-like and esterase (Kimura, Lopez and Salvaggio, 1975), amylase (Kuo and Hartman, 1966) and endopeptidase (Roberts *et al.*, 1977) activity in this organism. In addition, acid and alkaline phosphatase (Sinha and Singh, 1980) and lipase (Elwan *et al.*, 1978a; Elwan *et al.*, 1978b) has been shown. The presence of the latter three enzymes could not be found using the API ZYM strips. This could be a reflection of the assay substrates, the culture conditions, or isolate variation.

Table 4 Enzyme profile differences of thermophilic actinomycetes as detected by the API-ZYM system incubated at two temperatures

Enzyme detected	Incubation temperature	*T. candidus* (7)*	*T. vulgaris* (4)	*M. faeni* (2)
Alkaline phosphatase	50	+	–	+
	35	±	–	+
C8 esterase-lipase	50	+	–	+
	35	±	–	+
Cystine aminopeptidase	50	–	–	±
	35	–	–	–
Acid phosphatase	50	±	–	+
	35	–	–	–
Phosphoamidase	50	–	±	+
	35	–	–	+
α-glucosidase	50	–	+	–
	35	–	–	–
N-acetyl-β-glucosaminidase	50	–	–	–
	35	–	–	±

* Number of isolates tested.

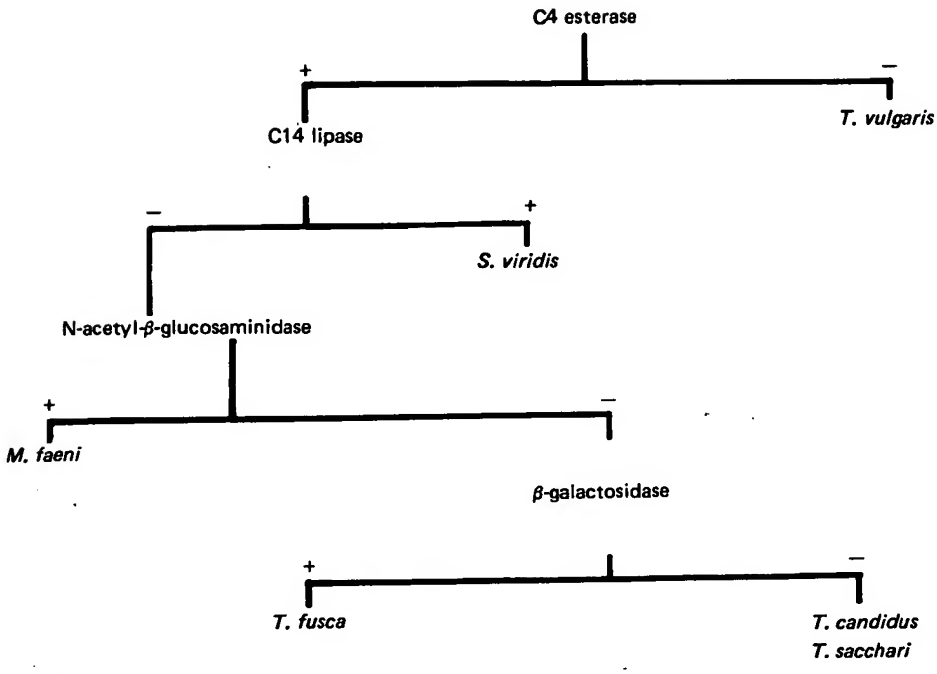

Figure 1 Generic identification involving a minimum of four test results using the API ZYM system.

M. faeni gave the greatest number of positive tests in the API ZYM strips. Positive reactions were noted in tests for acid and alkaline phosphatase, C4 and C8 esterase, leucine and cystine aminopeptidase, acid phosphatase, phosphoamidase, β-galactosidase and trypsin. The presence of these enzymes has been previously noted (Nicolet and Bannerman, 1975; Roberts *et al.*, 1977; Walbaum, Bigeut and Tran van Ky, 1969). Nicolet and Bannerman (1975) have also reported the isolation of several enzymes with chymotrypsin-like activity. The reason for our failure to detect this type of activity is not clear. However, the sensitivity of the commercial system may not be equivalent to assays described in the referenced research.

Few reports have appeared on the enzymatic activities of *T. candidus, S. viridis,* and *T. fusca.* Roberts *et al.* (1977) have described endopeptidases from *T. candidus,* but proteolytic activity was lacking for *S. viridis* and *T. sacchari.* Alkaline and acid phosphatase, phosphoamidase C4 and C8 esterase activity was demonstrated by *T. candidus* using the API ZYM system. It is possible that the esterase activity observed was actually due to a protease since it is known that certain proteases can cleave ester bonds (Stryer, 1975). In contrast to the results obtained by Roberts *et al.* (1977), *T. sacchari* showed alkaline phosphatase, C4 and C8 esterase-lipase activity, while *S. viridis* yielded alkaline phosphatase, C4 and C8 esterase-lipase, leucine aminopeptidase, β-galactosidase and α-glucosidase activities in the API ZYM system. *T. fusca,* which morphologically resembles *T. candidus* and *T. vulgaris,* could be differentiated from these actinomycetes by positive leucine aminopeptidase and β-galactosidase activities.

It would appear that the API ZYM strip compares favorably with conventional assay systems in that the majority of the enzymes which have been described for the thermophilic actinomycetes were also detected by this system. Kilian (1978) also found the strip to compare favorably with conventional assay systems.

In general, enzyme activity demonstrable at 50°C was also present at 35°C. Whether the enzymes not detected were inactive or had a low level of activity is not clear from this study. Although it would appear that most of the enzymes are extracellular (as revealed by the fact that they were found in the double dialysis preparation) liberation of the enzymes upon autolysis of the organism during the 2 week incubation period cannot be excluded. These data suggest that when assaying for the presence of antibody reactive with the enzymes in patients with hypersensitivity pneumonitis (HP), either the double dialysis antigen or extracts of whole cells can be used for *T. candidus* and *M. faeni*. However, to assess the reactivity of patients to the enzymes of *T. vulgaris* the double dialysis preparation should not be used since most of the enzymatic activity was found when whole cells were utilized.

The immunopathogenesis of HP has not been clearly elucidated (Roberts and Moore, 1977; Schatz, Patterson and Fink, 1977). Our previous data (Hollick, Hall and Larsh, 1979), as well as those of Burrell and Hill (1976) indicate that the disease cannot be reproduced in experimental animals by aerosol exposure alone. The findings of precipitating antibody in humans and animals directed against enzymes (Johnson *et al.*, 1980; Nicolet *et al.*, 1977; Nicolet, Bannerman and Krawinkler, 1974; Walbaum, Biguet and Tran van Ky, 1969), and the induction of HP in animals exposed to actinomycetes grown in hay (Burrell and Hill, 1976), suggests that enzymes may play a role in disease induction. Co-aerosolization with the intact bacterium and its associated enzymes may help to resolve this question.

The identification of the theromophilic actinomycetes is based primarily upon morphological and biochemical criteria (Kurup and Fink, 1975). Results from a large number of biochemical tests as well as resistance to heat and novobiocin are required for identification. Even with this *Thermoactinomyces* species can be difficult to distinguish from members of the genus *Thermomonospora*. The dichotomous key given in Figure 1 shows that generic identification is possible with a minimum of four test results using the API ZYM system. *T. fusca* gave positive tests for leucine aminopeptidase and β-galactosidase whereas none of the *Thermoactinomyces* species did so. Additionally, *T. vulgaris* and *T. candidus* are similar morphologically and biochemically. The data presented in Table 4 and Figure 1 show that the enzymatic profiles are quite distinct, and therefore, species identification is possible. Although *T. candidus* and *T. sacchari* gave identical enzymatic profiles these organisms differ morphologically so that misidentification should not occur.

In summary the differences in enzymatic profiles reported here for the thermophilic actinomycetes allowed clear differentiation between genera. The API ZYM system offers an easy and rapid assay system for nineteen different enzymes simultaneously.

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